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The nature of information, required for export and sorting, present within the outer membrane protein OmpA of *Escherichia coli* K-12

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Information, in addition to that provided by signal sequences, for translocation across the plasma membrane is thought to be present in exported proteins of *Escherichia coli*. Such information must also exist for the localization of such proteins. To determine the nature of this information, overlapping in-frame deletions have been constructed in the *ompA* gene which codes for a 325-residue major outer membrane protein. In addition, one deletion, encoding only the NH_2-terminal part of the protein up to residue 160, was prepared. The location of each product was determined by immunoelectron microscopy. Proteins missing residues 4–45, 43–84, 46–227, 86–227 or 160–325 of the mature protein were all efficiently translocated across the plasma membrane. The first two proteins were found in the outer membrane, the others in the periplasmic space. It has been proposed that export and sorting signals consist of relatively small amino acid sequences near the NH_2 terminus of an outer membrane protein. On the basis of sequence homologies it has also been suggested that such proteins possess a common sorting signal. The locations of the partially deleted proteins described here show that a unique export signal does not exist in the OmpA protein. The proposed common sorting signal spans residues 1–14 of OmpA. Since this region is not essential for routing the protein, the existence of a common sorting signal is doubtful. It is suggested that information both for export (if existent) and localization lies within protein conformation which for the former process should be present repeatedly in the polypeptide.

Key words: E. coli/immunoelectron microscopy/OmpA protein/outer membrane/protein export

Introduction

Proteins located in the outer membrane or the periplasmic space of *Escherichia coli* are usually synthesized in a precursor form possessing a typical prokaryotic signal sequence. There is undisputed evidence that these signal sequences are an absolute requirement for the export of such polypeptides and it also appears that a signal sequence alone does not suffice for translocation of a protein across the plasma membrane (Michaelis and Beckwith, 1982; Silhavy *et al.*, 1983; Kadanoga *et al.*, 1984). Furthermore, it has been shown that the localization of an outer membrane protein and a periplasmic protein is not determined by the signal sequence (Tommassen *et al.*, 1983; Ghrayeb *et al.*, 1984). As judged by the structures of signal sequences (Watson, 1984) this certainly is true for all outer membrane and periplasmic proteins. From these facts it has been concluded that information for export and proper localization are present in the mature part of an exported protein. Several types of experiments, mainly using *E. coli* outer membrane proteins, have indicated that such information does exist in the mature polypeptides. It has been suggested that it consists of short amino acid sequences, termed export and sorting signals, located at a unique region in a given protein (see Discussion). We have studied this problem with the 325-residue outer membrane protein OmpA of *E. coli* (Chen *et al.*, 1980) which is synthesized as a precursor with a 21-residue signal sequence (Beck and Bremer, 1980; Movva *et al.*, 1980). Here we show that in this protein an export signal of the type just mentioned does not exist. Our results also suggest that it is unlikely that a sorting signal as defined above is required for routing the protein to the outer membrane.

Results

Experimental approach

The general approach to the question of whether export or sorting signals exist in the mature OmpA protein was to construct overlapping deletions in the cloned gene and to determine the cellular location of the resulting polypeptides. The deletions were obtained in the cloned *ompA* gene in plasmid pTU 500 (Figure 1), as described in Materials and methods. The gene is under the control of the *lac* operator and promoter. Synthesis of OmpA from this gene results in the same cellular concentration of OmpA as that found in cells carrying chromosomally encoded wild-type *ompA*. The protein is not overproduced despite the presence of the gene on a high copy number plasmid because this gene carries a TAG stop codon corresponding to amino acid residue 7 of the protein; in the presence of the amber suppressor *supF* the limiting factor for translation is the concentration of the suppressor tRNA (Bremer *et al.*, 1980; Henning *et al.*, 1983).

Internal deletions

The polypeptides encoded by the internally deleted genes are schematically shown in Figure 2. The construction of the dele-

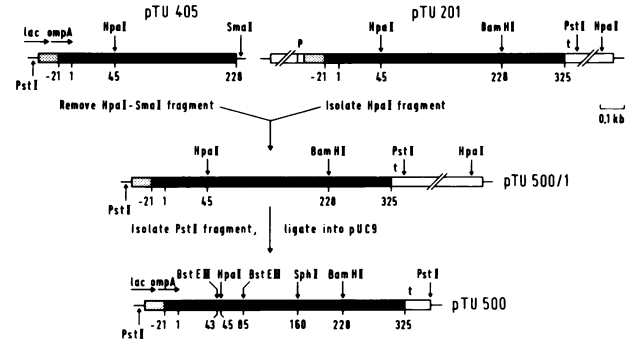

Fig. 1. Construction of plasmid pTU500. Thick lines represent the coding area of *ompA*, the stippled region the non-translated 5′ area of the gene, and the thin lines plasmid DNA. *lac*, promoter and operator of the lactose operon, p, t, promoter and terminator of the *ompA* gene, respectively. Numbers, amino acid positions in the OmpA protein (−21, translation start of the signal sequence). For details see Materials and methods.

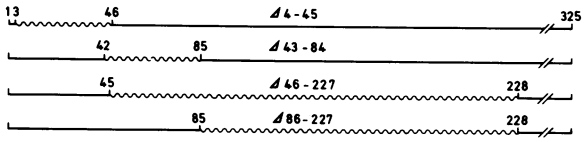

Fig. 2. Schematic presentation of deletions. Wavy lines indicate deleted DNA. The amino acid sequence at the area of the deletion in *ompA* Δ 86-227 is -Gly^{84}-Tyr^{85}-Gln^{44}-Val^{45}-Asp^{228}- (see Materials and methods). Numbers, amino acid positions in the OmpA protein.

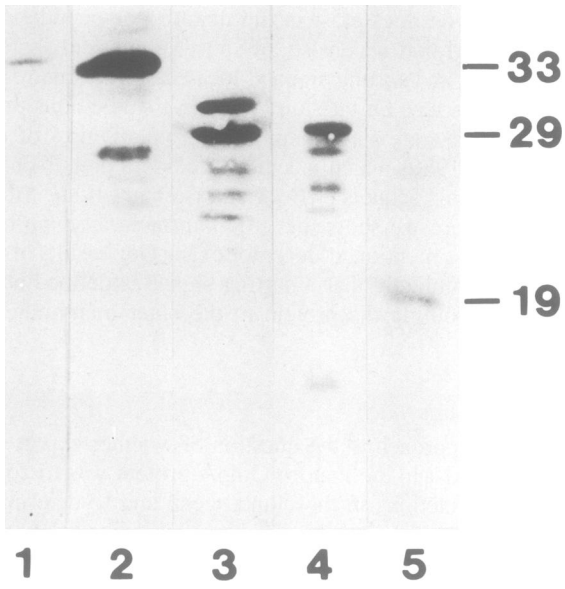

Fig. 3. Western blots of SDS-polyacrylamide gel electrophoretograms. Strain UH203 harboring plasmid pTU500 (**lanes 1, 2**), this plasmid carrying *ompA* Δ 4-45 (**lane 3**), *ompA* Δ 43-84 (**lane 4**) or *ompA* Δ 86-227 (**lane 5**) was grown in the presence of glucose (**lane 1**) or IPTG (**lanes 2–5**). The protein with the slowest mobility in **lane 3** is most likely its precursor; this polypeptide is overproduced (see text) and overproduction can cause accumulation of pro-OmpA protein (Henning *et al.*, 1979). An immunoblot is presented because although all gene products can also be stained some are difficult to discern from other proteins. Numbers, mol. wts. in kd.

tions, except *ompA* Δ 4-45, is described in Materials and methods.

Deletion *ompA* Δ 4-45 was found by accident during the isolation of lethal *ompA* mutants. In these experiments part of the gene corresponding to an area of the OmpA protein between amino acid residues −21 (start of the signal sequence) to 45 had been mutagenized and, among others, a mutant (*ompA* II-3e) was recovered in which several amino acid substitutions had occurred within the signal sequence and in which the NH_2 terminus of the mature protein is Val-Leu-Lys- instead of the wild-type sequence Ala-Pro-Lys-. This mutant protein is processed and incorporated into the outer membrane (Freudl *et al.*, 1985). It was noted that, upon induction of OmpA synthesis, one of these mutants was killed more rapidly than the others. DNA sequencing revealed that in this case (see Materials and methods) the plasmid contained an *ompA* gene with the same alterations as those of *ompA* II-3e but that, in addition, a deletion had occurred leading to the loss of amino acid residues from positions 4–45. The deletion removes the stop codon, in the *ompA* gene, described above. Overproduction of OmpA is lethal (Henning *et al.*, 1979; Bremer *et al.*, 1980). It is therefore understandable that expression of the *ompA* Δ 4-45 gene is rather toxic.

Gene products

Except for the deletion *ompA* Δ 46-227 the products of all internally deleted genes could be found in cell envelopes (Figure 3). They all exhibited the expected apparent mol. wts. Protein OmpA Δ 46-227 could not be detected by the Western blotting technique.

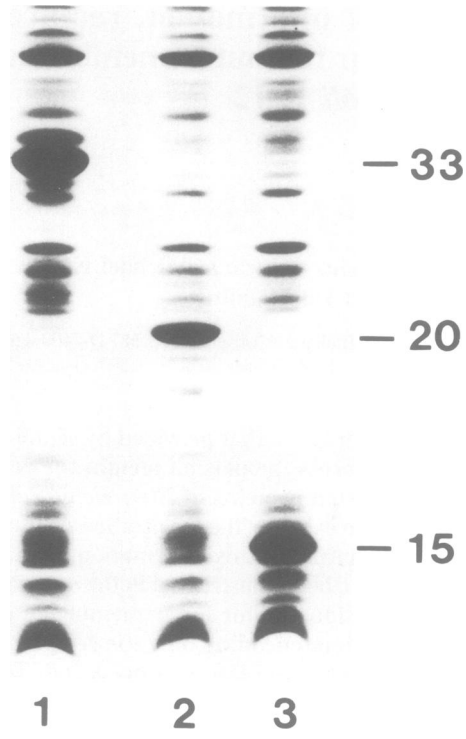

Fig. 4. Stained SDS-polyacrylamide gel electrophoretogram of cells. Strain UH203 harboring plasmids pRD87 (complete wild type *ompA* gene **lane 1**), pRD87 *ompA* Δ 160-325 (**lane 2**), or pRD87 *ompA* Δ 46-227 (**lane 3**) were pre-grown in medium containing glucose, washed once by centrifugation, diluted 1:1 in medium containing IPTG and incubated for 6 h. SDS extracts of whole cells were subjected to electrophoresis. Numbers, mol. wts. in kd.

A safe indicator of whether or not a secretory protein of *E. coli* has at least partially crossed the plasma membrane is removal of the signal sequence. Such processed proteins have never been detected in the cytoplasm. Pulse-chase experiments were performed with the deletion strains. In all cases, including *ompA* Δ 46-227, a precursor-product relationship was observed which is consistent with the interpretation that processing had occurred (data no shown). With *ompA* Δ 46-227, both the precursor and the mature protein disappeared after a chase time of 10 min. Obviously, the failure to detect the OmpA Δ 46-227 protein otherwise is due to rapid degradation of this polypeptide. We have asked if one can overcome this loss of the protein by increasing the concentration of the OmpA Δ 46-227 protein. For this purpose the deletion was prepared in plasmid pRD 87 (see Materials and methods) where the *ompA* gene does not possess a stop codon; the absence of this 'brake' leads to a rather massive overproduction of the OmpA protein. The protein could be found by staining electrophoretograms of induced cells (Figure 4).

Cellular location of internally deleted OmpA proteins

Results obtained from cell fractionation experiments often yielded ambiguous results when applied to altered OmpA proteins (unpublished). We have therefore used electron microscopy of immuno-gold labeled thin sections of cells embedded in Lowicryl K4M (Figure 5) to locate the protein products. As expected, the OmpA Δ 46-227 protein could not be detected. Clearly, those of deletions *ompA* Δ 4-45 and *ompA* Δ 43-84 were exclusively associated with the outer membrane. The OmpA Δ 86-227 pro-

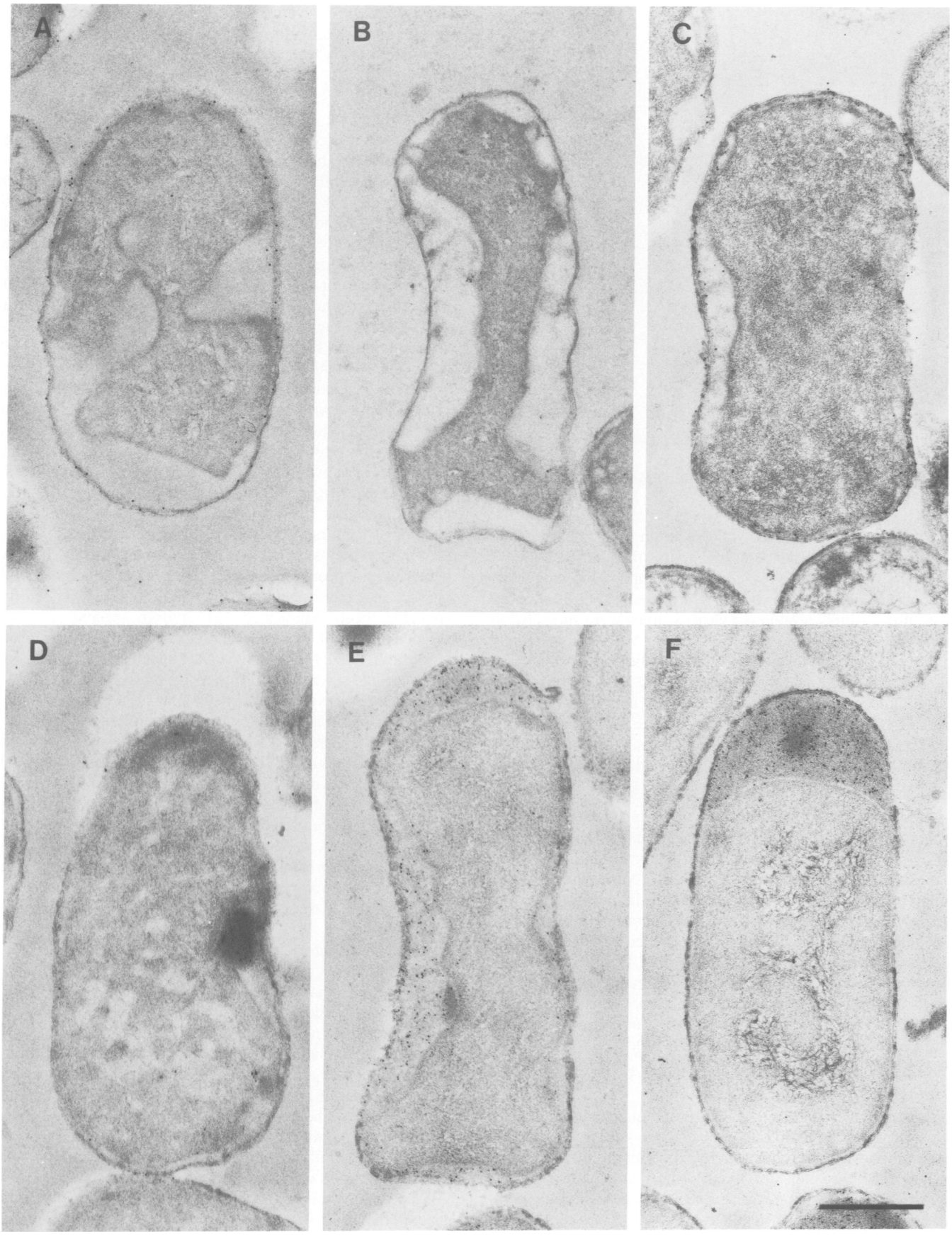

Fig. 5. Immunocytochemical localization of gene products in ultrathin sections. **A**, *ompA*$^+$ parent of strain UH203; **B**, strain UH203. **C**–**F**, strain UH203 carrying plasmid pTU500 with *ompA* Δ 4-45 (**C**, exactly the same pictures were obtained with *ompA* Δ 43-84) or *ompA* Δ 86-227 (**D**). The same strain harboring plasmid pRD87 with *ompA* Δ 46-227 after 2 h induction with IPTG (**E**) and after 4 h induction (**F**). Bar represents 0.5 μM.

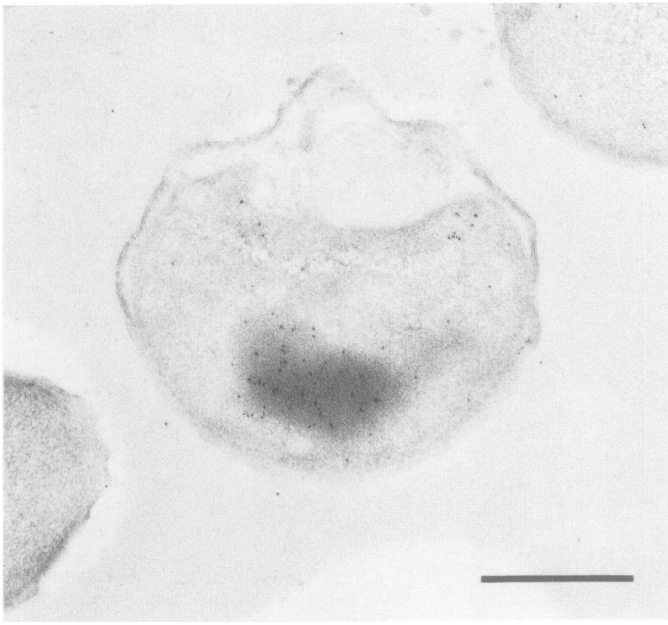

Fig. 6. Immunocytochemical localization of the OmpA Δ-15-45 protein. Bar as in Figure 5.

tein was found in the periplasmic space in large clumps, i.e., in a precipitated state. As described above, the OmpA Δ 46-227 protein could be detected by staining electrophoretograms when the concentration of the product was increased. Cells induced for synthesis of this protein for various times were subjected to the immuno-gold labeling technique. Figure 5 shows that initially the protein is present evenly distributed in the periplasmic space and that later the same precipitates develop as described above for the OmpA Δ 86-227 protein. Clearly, and consistent with the pulse-chase experiment, the Omp Δ 46-227 protein is efficiently exported to the periplasmic space, but apparently cannot be incorporated into the outer membrane and finally precipitates.

A COOH-terminal deletion, ompA Δ 160-325

We have reported earlier on a truncated *ompA* gene, encoding an OmpA protein of 160 NH_2-terminal residues (Henning *et al.*, 1983). The product of this gene could not be found in cells. We have asked if in this case one can also overcome the loss of the protein by increasing the concentration of the polypeptide. The deletion was therefore constructed in the *ompA* gene without the stop codon in plasmid pRD87. The product was detectable by staining electrophoretograms of envelopes from induced cells (Figure 4). Immunoelectron microscopy revealed that the protein was present only in the periplasmic space (not shown, electron micrographs are indistinguishable from those shown in Figure 5 for the OmpA Δ 46-227 protein).

A control: an internal deletion leaving the gene product in the cytoplasm

All gene products described so far were found either in the outer membrane or in the periplasm. Hence the possibility existed that one or other of these products were exported only partially, that a major fraction remained in the cytoplasm and that the method used would not detect an OmpA protein present in the latter compartment. Therefore, the deletion *ompA* Δ-15-45 was constructed. It removes residues −1 to −15 of the signal sequence plus residues 1−45 of the mature protein (see Materials and methods). The expected cytoplasmic location of the OmpA Δ-15-45 protein could clearly be demonstrated by the immuno-gold labeling technique (Figure 6). The protein, mostly in an aggregated state, is present in large amounts because the deletion removes the stop codon in the gene (see above).

Discussion

Detailed studies concerning the question addressed in this communication have been performed with the *E. coli* outer membrane protein LamB (Hall *et al.*, 1982; Benson and Silhavy, 1983; Benson *et al.*, 1984). It was concluded that, within this protein, sequence information exists which is required for export and, perhaps separately, for incorporation into the outer membrane. The cellular locations of hybrid proteins, consisting NH_2-terminally of small parts of the LamB protein and COOH-terminally of the large cytosolic β-galactosidase, were determined. The results suggested that a signal for export is present between amino acid residues 27 and 43 and that a least part of a sorting signal, routing the hybrids to the outer membrane, is present between residues 39 and 49 (Benson *et al.*, 1984). From results obtained with *ompA-lacZ* fusions it was also suggested that a small NH_2-terminal segment of OmpA contains the signal necessary for export (Palva and Silhavy, 1984). Computer analyses have shown that the area of the sorting signal exhibits homology with regions, near their NH_2 termini, of five other outer membrane proteins (Nikaido and Wu, 1984). This homology, of course, appeared to support the proposal that outer membrane proteins contain a common sorting signal. The outer membrane lipoprotein does not possess an area homologous to this sequence but the presence, in this protein, of a short signal has also been implicated. A hybrid protein consisting NH_2-terminally of 11 residues of the outer membrane protein OmpF and COOH-terminally of the outer membrane lipoprotein (missing seven NH_2-terminal residues) was found in the periplasmic space (Yu *et al.*, 1984), while a hybrid consisting of nine NH_2-terminal residues of the lipoprotein followed by the periplasmic β-lactamase was localized to the outer membrane (Ghrayeb and Inouye, 1984). It is not excluded, however, that the determination of the location of some of the hybrid proteins may have been influenced by artefacts. In all of these studies this location has been determined by cell fractionation experiments and it has recently been shown that such techniques, when applied to a hybrid protein consisting of 300 NH_2-terminal residues of the outer membrane protein PhoE fused to an almost complete β-galactosidase, can lead to artificial results (Tommassen *et al.*, 1985).

What sort of information for export and localization is present in the OmpA protein? From earlier studies we know that the COOH-terminal moiety of OmpA, starting around residue 190, is not required for the proper incorporation of the protein into the outer membrane (Bremer *et al.*, 1982; Henning *et al.*, 1983). Here we have shown that for translocation across the plasma membrane a specific signal, i.e., an amino acid sequence at a unique site of the protein, does not exist between residues 4 and 228. Also, the first three residues cannot specifically signal export because in the OmpA Δ 4-45 protein this sequence is Val-Leu-Lys- instead of the wild-type sequence Ala-Pro-Lys-. Thus, one specific export signal does not exist in the mature polypeptide.

We believe that it is not absolutely clear whether information for export is present in mature secreted proteins of *E. coli*. In the cases reported where signal sequences are unable to cause export, lack of export need not necessarily be due to missing information but possibly to inhibition of translocation. If such information does exist in the OmpA protein, the only possible

'signal' which we can envisage is one in which protein conformation dictates translocation across the plasma membrane. For the arrangement of the OmpA protein in the outer membrane we have proposed a model in which residues 1 – 177 of OmpA cross the membrane eight times in cross-β sheet conformation (Morona *et al.*, 1984). Such an arrangement has a conformationally repetitive character. One way of understanding the nature of the information required for export of the OmpA protein is based on this repetitiveness. In such a model, it would suffice that a domain (e.g., one of the four loops) of the protein folds in a certain way to 'induce' translocation across the plasma membrane, and one such domain then could replace another if they assume similar conformations. A requirement for a certain conformation to trigger export is also consistent with the fact that to be processed *in vivo*, the OmpA protein has to reach a critical mol. wt. of ~30 000; i.e., ~86% of the protein is synthesized before processing begins (Josefsson and Randall, 1981; see also the review by Randall and Hardy, 1984).

What features of the OmpA protein determine its localization? In this protein the area of homology suggested to contain the sorting signal common to outer membrane proteins spans residues 1 – 14. Protein OmpA Δ 4-45 was found only associated with the outer membrane. Therefore, this area cannot be essential for directing the protein to its membrane and it appears doubtful that a common sorting signal exists for outer membrane proteins. Proteins OmpA Δ 46-227, OmpA Δ 86-227 and OmpA Δ 160-325 remain periplasmic. It is possible that a sorting signal of the type discussed initially is missing in these proteins. If so, it should be located between amino acid residues 160 and 193 or, more likely, between residues 160 and 177. The 193 NH_2-terminal residues suffice to bring the truncated polypeptides to the outer membrane (Henning *et al.*, 1983). It is known that in native OmpA residue 177 is located in the periplasm (Chen *et al.*, 1980) and, thus, it is unlikely that the region between residues 177 and 193 has anything to do with sorting. If a sorting signal is present between residues 160 and 177 it does not constitute a sorting signal common to outer membrane proteins (Nikaido and Wu, 1984). We believe, however, that for the following reason it is unlikely that this potential signal exists. The presence of the three partially deleted proteins in the periplasmic space suggests that the wild-type OmpA protein is also transiently present in this compartment. This assumption is supported by the demonstration that processed OmpA protein could, although under non-physiological conditions, be found in the periplasm (as defined by osmotic shock) and could be chased into the outer membrane (Halegoua and Inouye, 1979). If the normal route of export of OmpA is via the periplasm (such a route does not imply that it diffuses through this space, the periplasmic species could be extrinsic to both the plasma and the outer membrane) it appears rather unlikely that a short amino acid sequence as such serves as a sorting signal. A more attractive possibility is that localization is ruled by protein conformation. Experiments to be reported elsewhere have shown that indeed a processed OmpA protein could be found, although under non-physiological conditions, in the periplasmic space and that this fraction of the polypeptide differs in conformation from the one present in the outer membrane.

Materials and methods

Bacterial strain and growth conditions

The host for all plasmids was the *E. coli* K-12 strain UH203 which is *lac, supF, ompA* (not producing the OmpA protein), *recA, proA* or *B, rpsL*, F' lacI^Q, lacZΔ M15 *proAB*$^+$ (Freudl *et al.*, 1985). Cells were grown at 37°C in L-broth (Miller, 1972) supplemented with ampicillin (20 μg/ml) and, for induction of OmpA synthesis, with isopropylthiogalactoside (1 mM). Glucose (0.5%) was added when the expression of an *ompA* gene was toxic.

Construction of plasmids and ompA deletions

The construction of plasmid pTU405 (Figure 1) was described previously (Freudl *et al.*, 1985). In brief, a promoterless *ompA* fragment containing the non-translated 5' area plus the coding region for the first 228 amino acid residues was ligated into plasmid pUC9 (Vieira and Messing, 1982) in an orientation placing the fragment under the control of the *lac* promoter and operator present on this plasmid. The inducible *ompA* gene was used to identify mutants which cause lethality when the altered genes are expressed. We used an inducible gene system, as the possibility existed that expression of internally deleted *ompA* genes might be deleterious. To avoid possible side effects created by the presence of the truncated OmpA in the outer membrane we also completed the gene (Figure 1). A 2-kd *Hpa*I fragment from plasmid pTU 201, consisting of a 7.5-kd chromosomal *Eco*RI fragment in pBR325 (Bremer *et al.*, 1980), was ligated into pTU405 deleted for the *Hpa*I-*Sma*I area yielding plasmid pTU500/1. Because this plasmid contains undesired restriction sites to the right of *ompA*, the 1.28-kb *Pst*I fragment was ligated into the *Pst*I site of pUC9, resulting in plasmid pTU500. For the experiments requiring an inducible *ompA* gene not possessing the stop codon mentioned in the Results section plasmid pRD87 was prepared. It has the same structure as pTU500 (Figure 1) except that the final cloning vector was pUC8 (Vieira and Messing, 1982). A site for *Acc*I is located between the *ompA* gene and its promoter (Cole *et al.*, 1982). Plasmid pTU100 (Bremer *et al.*, 1980) harboring the *ompA* gene without the amber codon, was digested with *Acc*I and the *Acc*I sites were filled in with the Klenow fragment of DNA polymerase I, followed by cleavage with *Pst*I. The isolated 1.285-kb *Acc*I-*Pst*I fragment (the *Pst*I site is the same as this distal site in pTU500) was ligated into pUC9 opened with *Sma*I and *Pst*I. The resulting plasmid contains the *ompA* gene in an orientation opposite to the *lac* promoter. It was placed under *lac* control by isolating the *Eco*RI-*Hind*III fragment and ligating it into pUC8 restricted with the same enzymes. In this plasmid, pRD87, the deletion *ompA* Δ 160-325 was obtained by first removing the *Eco*RI site in the plasmid (cleavage, filling in and religation), then removing the *Sph*I-*Pst*I fragment followed by treatment with S1 nuclease. Because religation would cause the *ompA* reading frame to continue considerably into the plasmid, a 12-mer *Eco*RI linker was introduced at this site. Restriction with *Eco*RI, filling in and religation created a TAA stop codon two amino acid residues away from residue 160 of the OmpA protein.

Deletion *ompA* Δ 43-84 was produced by removing the 0.125-kb *Bst*EII fragment of the gene followed by religation. The deletion *ompA* Δ 46-227 was obtained by removing the 0.545-kb *Hpa*I-*Bam*HI fragment, filling in the *Bam*HI site and religation. In this gene the proximal *Bst*EII site is still present and the 0.125-kb *Bst*EII fragment was ligated into this site resulting in *ompA* Δ 86-227. Because of the closely neighbored *Bst*EII and *Hpa*I sites two amino acid residues between the two sites are not eliminated by this operation and the sequence at the area of the deletion is -Gly^{84}-Tyr^{85}-Gln^{44}-Val^{45}-Asp^{228}-. The deletion *ompA* Δ 46-227 was also obtained in plasmid pRD87. For the preparation of deletion *ompA* Δ-15-45 a unique *Nru*I site was utilized which in plasmid pTU500 is present within the codon for amino acid residue – 16 of the OmpA signal sequence. Cleavage of the plasmid with *Nru*I and *Hpa*I, removal of the small *Nru*I-*Hpa*I fragment and ligation in the presence of an 8-mer *Bam*HI linker resulted in an *ompA* gene containing the sequence –ATC GCG GAT CCG AAC- (linker sequence underlined) encoding -Ile^{-16}-Ala-Asp-Pro-Asn^{46}-. The 21-residue signal sequence was thus reduced to Met^{-21}-Lys-Lys-Thr-Ala-Ile^{-16}-. All restriction fragments were isolated by agarose gel electrophoresis followed by electroelution (Yang *et al.*, 1979). The identity of the deletions was proven by the absence or presence of relevant restriction sites, the sizes of the deleted *ompA* genes, and the apparent mol. wts. of the gene products.

The deletion *ompA* Δ 4-45 was identified by DNA sequencing using the dideoxy chain-termination method (Sanger *et al.*, 1977). The plasmid, possessing only the distal *Bst*EII site (see Figure 1), was cleaved with this enzyme and the ends were filled in (see above). The small fragment then obtained by cleavage with *Pst*I was ligated into phages M13 mp9 and mp8 (Messing and Vieira, 1982) opened with *Pst*I and *Sma*I. Phage DNA was sequenced using the universal primer (New England Biolabs). The enzymes were from Boehringer, Mannheim or New England Biolabs. In detail, all manipulations were performed essentially as described by Maniatis *et al.* (1982).

Identification of gene products

The pulse-chase and Western blotting experiments were performed as described previously (Freudl *et al.*, 1985). For electrophoresis Laemmli type gels (Laemmli, 1970) with 12% acrylamide were used.

Immunoelectron microscopy

For better differentiation of inner and outer membranes, cells were plasmolyzed with 25% sucrose before fixation with 2% formaldehyde and 0.05% glutaraldehyde. (Plasmolysis does not create artefacts; the same results were ob-

tained without the treatment with sucrose, but much more effort was required to find cells in which the two membranes were sufficiently separated.) After embedding the cells in agarose the samples were dehydrated and embedded in Lowicryl K4M at low temperature (Carlemalm *et al.*, 1982). Ultrathin sections were treated with rabbit OmpA antiserum and protein A-gold complexes prepared with tannic acid (Mühlpfordt, 1982). The sections were examined in a Philips 201 electronmicroscope at 60 kV. As already evident from the electron micrographs there is essentially no background label. We have determined this label by counting grains inside (cytoplasm) and outside cells in all samples corresponding to those shown in Figure 5. A total area of 499.5 μm^2 was covered and 0.96 $\pm$ 0.3 grains/μm^2 were found which is negligible and hence cannot possibly blur the results.

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